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Biochemistry

Involvement of the cell-cycle inhibitor *Cip1/WAF1* and the E1A-associated p300 protein in terminal differentiation

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ABSTRACT **The mechanism of cell cycle withdrawal during terminal differentiation is poorly understood. We report here that the cyclin-dependent kinase (CDK) inhibitor $p21^{Cip1/WAF1}$ is induced at early times of both keratinocyte and myoblast differentiation. $p21^{Cip1/WAF1}$ induction is accompanied by a drastic inhibition of total Cdk2, as well as $p21^{Cip1/WAF1}$-associated CDK kinase activities. $p21^{Cip1/WAF1}$ has been implicated in p53-mediated G_1 arrest and apoptosis. In keratinocyte differentiation, *Cip1/WAF1* induction is observed even in cells derived from p53-null mice. Similarly, keratinocyte differentiation is associated with induction of *Cip1/WAF1* promoter activity in both wild-type and p53-negative keratinocytes. Induction of the *Cip1/WAF1* promoter upon differentiation is abolished by expression of an adenovirus E1A oncoprotein (dl922/947), which is unable to bind p105-Rb, p107, or cyclin A but which still binds the nuclear phosphoprotein p300. Overexpression of p300 can suppress the E1A effect, independent of its direct binding to E1A. Thus, terminal differentiation-induced growth arrest in both keratinocyte and myoblast systems is associated with induction of *Cip1/WAF1* expression. During keratinocyte differentiation, *Cip1/WAF1* induction does not require p53 but depends on the transcriptional modulator p300.**

The mechanisms responsible for cell cycle arrest in terminally differentiating cells are poorly understood. Progression through the cell cycle is controlled by activation of a series of cyclin-dependent kinases (CDKs; ref. 1). In addition to positive regulation by cyclins, CDK activity is regulated by phosphorylation or dephosphorylation at specific residues, as well as by association with a number of inhibitory proteins (2). Among these, a 21-kDa protein ($p21^{Cip1/WAF1}$) has been identified, which binds in quaternary complexes with CDK kinases, their associated cyclins, and the proliferating cell nuclear antigen (PCNA; refs. 3 and 4). Inhibition of CDK activity by $p21^{Cip1/WAF1}$ depends on the relative stoichiometric amounts of this inhibitor in the complex (5). Besides regulating CDK activity, $p21^{Cip1/WAF1}$ can directly inhibit DNA replication by blocking the ability of PCNA to activate DNA polymerase δ (6, 7).

Induction of *Cip1/WAF1* gene expression has been previously implicated in p53-mediated growth arrest and apoptosis (8, 9), as well as cellular senescence, quiescence, transient growth-factor stimulation, and pharmacologically induced differentiation of hematopoietic and hepatoma cells (10–13). Mechanisms for the modulation of *Cip1/WAF1* gene expression other than by p53 activation have not been previously described.

The adenovirus E1A oncoprotein provides a useful tool to interfere with key cellular proteins involved in transcriptional and DNA synthesis control, cell cycle, and differentiation. The amino terminal region of E1A is required for transformation (14) and is responsible for repression of a number of viral enhancers, as well as enhancers and promoters of tissue-specific genes induced during differentiation (15). This region of E1A is characterized by the ability to bind and presumably inactivate a large nuclear phosphoprotein, p300 (15, 16). This protein contains a bromodomain, a hallmark of several transcriptional coactivators, as well as three cysteine/histidine rich regions (16). The most carboxyl terminal of these regions is involved in the specific binding to E1A. Overexpression of p300 or a p300 mutant lacking the E1A-binding region can counteract the inhibitory effects of E1A on the simian virus 40 enhancer, suggesting that p300 may function as a coactivator molecule at complex transcriptional regulatory sites (16).

We report here that $p21^{Cip1/WAF1}$ expression is induced at early times of keratinocyte and myoblast differentiation in parallel with a strong decrease in total CDK2 and p21-associated CDK activity. Induction of $p21^{Cip1/WAF1}$ expression in differentiation is unlinked from p53 control, while it depends on a p300-dependent mechanism. Thus, a connection has been established between this transcriptional modulator and a differentiation signal linked to growth arrest.

MATERIALS AND METHODS

Cell Culture. Primary keratinocytes from newborn Sencar mice were cultivated in low-calcium (0.05 mM) medium as described (17, 21). For all experiments, cells were used 1 week after plating. Differentiation was induced by addition of 2 mM calcium chloride.

Primary keratinocytes lacking the p53 gene were obtained from newborn, p53-null mice (18).

C2C12 mouse myoblasts grown in 20% (vol/vol) fetal calf serum in Dulbecco's modified Eagle's medium (DMEM) were induced to differentiate by changing the medium to DMEM containing 5% (vol/vol) horse serum (19).

Northern Hybridization Analysis. Total RNA from keratinocytes and C2C12 myoblasts (20) was fractionated on a 1.2% agarose/formaldehyde gel, transferred to Hybond N+ membrane (Amersham), and probed with a ^{32}P-labeled mouse *Cip1/WAF1* cDNA fragment [corresponding to 300 bp of the published sequence (8)].

Antibodies and Immunoprecipitation Experiments. Cells were labeled with 0.1 mCi of [^{35}S]methionine/cysteine (1 Ci = 37 GBq; Expre^{35}S^{35}S; NEN) per ml for 4 h in methionine-free medium containing serum. Cells were lysed in 250 mM NaCl, 50 mM Tris·HCl, pH 7.4/5 mM EDTA/0.1% Triton X-100, in the presence of protease and phosphatase inhibitors. Samples were precleared with rabbit serum coupled to protein A-

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Abbreviations: CDK, cyclin-dependent kinase; TGF-β, transforming growth factor β; pRb, retinoblastoma protein; GST, glutathione *S*-transferase; PCNA, proliferating cell nuclear antigen.

Sepharose (Pharmacia) and normalized for amounts of radioactivity. Immunoprecipitations were carried out overnight with polyclonal antibodies against murine $p21^{Cip1/WAF1}$ (provided by C. Schneider; similar results were obtained with antibodies purchased from PharMingen) or with normal rabbit preimmune serum, as a control.

p300 immunoprecipitation experiments were performed in the same lysis buffer by using anti-p300 monoclonal antibodies (1:1 mixture of RW105 and RW128; ref. 16). Immunocomplexes were incubated with protein G-agarose (Boehringer). Phosphatase digestion was as described (21).

For p53 immunoprecipitation, monoclonal antibodies against wild-type murine p53 (PAb246; Oncogene Science) were used.

Kinase Assays. Immunoprecipitations were performed as described above, but cells were lysed in 150 mM NaCl/50 mM Tris·HCl, pH 7.4/5 mM EDTA/0.1% Tween 20. After immunoprecipitation, samples were washed four times with lysis buffer and two times in kinase buffer (50 mM Hepes, pH 7.4/10 mM $MgCl_2$/5 mM $MnCl_2$/1 mM dithiothreitol), and then resuspended in 50 μl of kinase buffer containing 5 μCi of [^{32}P]ATP (6000 Ci/mmol; NEN) and 1 μM ATP (Pharmacia). Samples were incubated for 30 min at 30°C in the presence of either 5 μg of histone H1 (Boehringer) or 0.2 μg of soluble glutathione *S*-transferase (GST)–retinoblastoma protein (pRb) (22). Reactions were analyzed by SDS/10% PAGE and autoradiography.

Transient Transfections and Luciferase Assays. Primary keratinocytes 5 days after being plated were transfected with plasmid DNA by the DEAE-dextran technique (23) in serum-free, low-calcium medium for 2 h, followed by dimethyl sulfoxide shock for 1 min. The SV2neo plasmid was used as carrier so that all dishes received the same amount of total DNA (20 μg of DNA per 100-mm dish). Unless otherwise indicated, transfected keratinocytes were either kept in low-calcium medium or exposed to high calcium concentrations for the last 24 h of a 72-h incubation period. Cells were harvested in all cases at 72 h and luciferase assays were performed as described (25). Values were normalized for total protein concentrations and expressed either as cpm $\times 10^{-4}$ per 10 μg of cell extract or as fold induction relative to basal promoter activity.

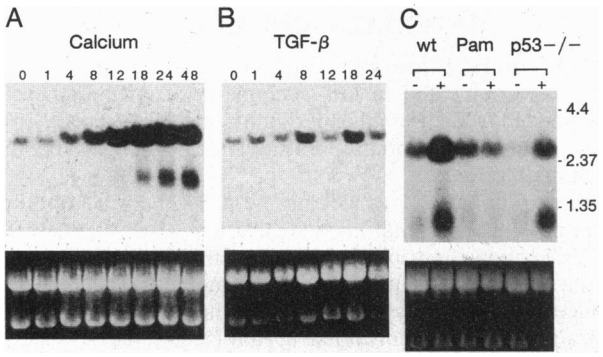

FIG. 1. Induction of *Cip1/WAF1* mRNA expression in calcium-induced keratinocyte differentiation. (*A* and *B*) Northern blot analysis using a mouse *Cip1/WAF1*-specific probe of total cellular RNA (30 μg per sample) from mouse primary keratinocytes under growing conditions (time 0) and at various times (in hours) after exposure to calcium (2 mM) (*A*) or TGF-β (3 ng/ml) (*B*). Specific induction of *Cip1/WAF1* expression in response to calcium was confirmed by Northern and ribonuclease protection assays in three other independent experiments. (*C*) Northern blot analysis using the *Cip1/WAF1*-specific probe of total RNA (20 μg per sample) from wild-type mouse primary keratinocytes, calcium-resistant Pam212 keratinocytes, or primary keratinocytes derived from p53-null mice. Cells were either maintained in medium at low calcium concentrations (0.05 mM) (−) or switched to high calcium concentrations (2 mM) for 24 h (+). The position of RNA molecular weight markers (GIBCO/BRL) is indicated. The lower panels are ethidium bromide-stained agarose gels before the Northern transfer to show the concentration and integrity of the various RNA samples.

RESULTS

Induction of *Cip1/WAF1* Expression in Keratinocyte Differentiation. Addition of calcium to mouse primary keratinocyte cultures triggers a terminal differentiation program very similar to that observed in the upper epidermal layers *in vivo*. This includes induction of a number of biochemical and morphological markers, as well as growth arrest (17). In contrast to calcium, exposure of primary keratinocytes to transforming growth factor β (TGF-β) results in inhibition of cell growth but does not induce any differentiation markers (ref. 26 and our unpublished observations).

In a series of studies on growth-arrest mechanism associated with keratinocyte differentiation, we measured *Cip1/WAF1* expression after treatment with calcium or TGF-β. As shown in Fig. 1*A*, *Cip1/WAF1* mRNA was induced as early as 4 h after calcium exposure, and its expression progressively increased up to 18–24 h, at which time DNA synthesis is almost totally inhibited (17). *Cip1/WAF1* induction was specifically associated with differentiation-induced growth arrest, as it was not observed after exposure of keratinocytes to TGF-β (Fig. 1*B*).

The Pam212 keratinocyte line is resistant to calcium-induced differentiation, while retaining sensitivity to TGF-β-induced growth inhibition (24). Unlike primary keratinocytes, no induction of *Cip1/WAF1* expression was observed with

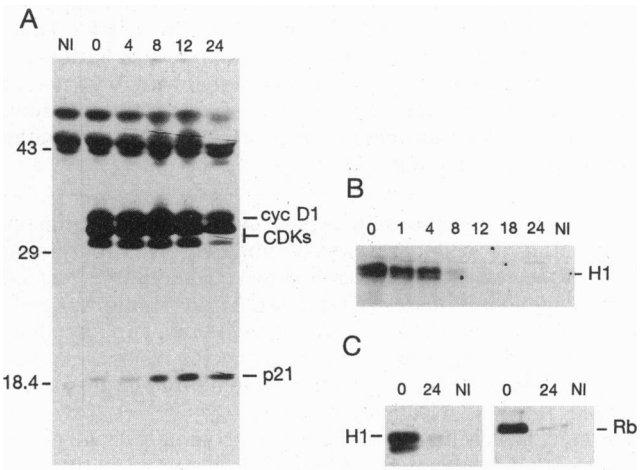

FIG. 2. Induction of $p21^{Cip1/WAF1}$ expression and inhibition of total CDK2 and p21-associated CDK activity in differentiating keratinocytes. (*A*) Immunoprecipitation with anti-$p21^{Cip1/WAF1}$ polyclonal antibodies of keratinocyte lysates that were either kept under growing conditions (time 0) or induced to differentiate by increasing the calcium for the indicated amounts of time (in hours). Keratinocytes were labeled with [^{35}S]methionine/cysteine for the last 4 h prior to being harvested. Immunoprecipitates were analyzed by SDS/12% PAGE and fluorography. The position of molecular size markers is indicated on the left, and the position of p21 and p21-associated proteins is indicated on the right. Identification of the latter proteins was obtained in preliminary experiments by parallel immunoprecipitations with p21-, CDK2-, CDK4-, and cyclin D1 (cyc D1)-specific antibodies. (*B*) *In vitro* CDK2 kinase assays with extracts from keratinocytes under growing conditions and at various times (in hours) of calcium-induced differentiation. The position of the histone H1, which was used as a substrate, is indicated. (*C*) *In vitro* CDK kinase assays with anti-$p21^{Cip1/WAF1}$ immunoprecipitates from keratinocyte lysates from cells cultured either under growing conditions (time 0) or after 24 h of calcium-induced differentiation. Histone H1 or purified GST–pRb (Rb) protein was used as the substrate. NI, nonimmune control.

Pam212 cells grown in medium at low calcium concentrations for 1 week and exposed to high calcium concentrations for 24 h (Fig. 1*C*).

Immunoprecipitation of [^{35}S]methionine/cysteine-labeled keratinocyte lysates with anti-p21$^{Cip1/WAF1}$ antiserum revealed increasing amounts of labeled p21$^{Cip1/WAF1}$ protein by 8 h of calcium-induced differentiation (Fig. 2*A*). That this increase reflects an increase of total p21 protein levels was indicated by a pulse–chase experiment showing that the stability of the p21 protein is not significantly affected during differentiation, as well as by direct immunoblotting (up to 12 h; data not shown). Among the p21-associated proteins, cyclin D1 levels remained constant, while CDK levels did not change at early times of keratinocyte differentiation but were somewhat decreased by 24 h (Fig. 2*A*).

Total CDK2 kinase activity was drastically reduced as early as 8 h after induction of differentiation (Fig. 2*B*). Inhibition of CDK activity was also found in anti-p21 immunoprecipitates by using either histone or GST–pRb as substrates (Fig. 2*C*).

Induction of *Cip1/WAF1* Expression in Myoblast Differentiation. The myoblast to myotube conversion provides another well-characterized differentiation system. We found that induction of *Cip1/WAF1* mRNA expression also occurs in this system. *Cip1/WAF1* mRNA levels were strongly induced 24 h after inducing myoblast differentiation and further increased by 48 and 72 h (Fig. 3*A*). Levels of the p21$^{Cip1/WAF1}$ protein were also increased by 48 h of differentiation as detected by either immunoprecipitation with anti-p21 antibodies (Fig. 3*B*) or direct immunoblotting (not shown). Amounts of p21-associated cyclin D1 remained constant, while those of p21-associated CDKs seemed to increase (Fig. 3*B*). Even in this case, total CDK2 kinase activity was drastically reduced after induction of differentiation (Fig. 3*C*).

Thus, induction of p21$^{Cip1/WAF1}$ expression is observed in both keratinocyte and myoblast differentiations, in parallel with a block of total CDK2 and p21-associated CDK activity.

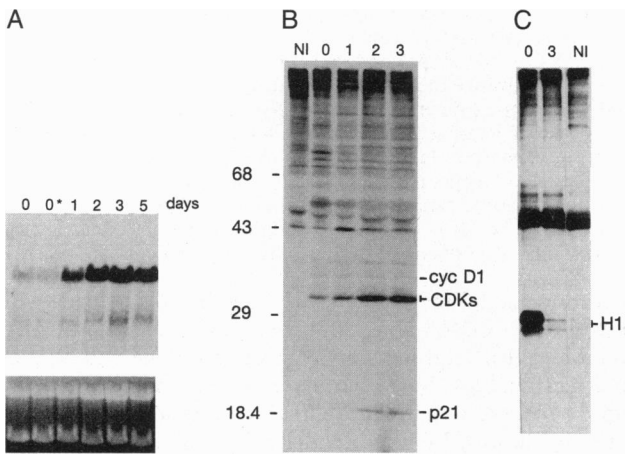

FIG. 3. Induction of *Cip1/WAF1* expression and decreased CDK kinase activity in myoblast differentiation. (*A*) Northern blot analysis using the *Cip1/WAF1*-specific probe of total RNA (20 μg per sample) from C2C12 myoblasts which were either kept in growth medium under subconfluent (0) or freshly confluent conditions (0*), or were induced to differentiate into multinucleated myotubes by switching to differentiation medium for the indicated amounts of time (in days). The lower panel is the ethidium bromide stained agarose gel before Northern transfer. (*B*) Immunoprecipitation with anti-p21$^{Cip1/WAF1}$ of [^{35}S]methionine/cysteine-labeled C2C12 myoblast lysates prepared from cells under growing conditions or at various times (in days) after induction of differentiation. The positions for the p21$^{Cip1/WAF1}$ protein, CDKs, and cyclin D1 (cyc D1) are indicated. (*C*) *In vitro* CDK2 kinase assays with extracts from C2C12 myoblasts under growing conditions and at 3 days after induction of differentiation. The position of the histone H1 substrate is indicated. NI, nonimmune control.

Induction of *Cip1/WAF1* Expression in Keratinocyte Differentiation Is Unlinked from p53 Control. *Cip1/WAF1* expression was previously reported to be under p53 control (8, 9). p53 protein expression was found to decrease in primary keratinocytes induced to differentiate by calcium (Fig. 4*A*). To determine conclusively whether p53 protein is required for induction of *Cip1/WAF1* expression, primary keratinocytes were derived from p53-null mice. Basal levels of *Cip1/WAF1* mRNA were found to be lower in these cells. However, even in p53-null keratinocytes, calcium treatment caused a significant *Cip1/WAF1* mRNA induction (Fig. 1*C*).

To study further the mechanism of *Cip1/WAF1* induction during keratinocyte differentiation, we transiently transfected mouse primary keratinocytes with a 2.4-kb DNA fragment of the human *Cip1/WAF1* promoter linked to a luciferase reporter gene (WWP-luc; ref. 8). Induction of this promoter increased progressively up to 24 h (Fig. 5*A*). A *Cip1/WAF1* promoter lacking the fully conserved p53 recognition site at its 5′ end (DM-luc; ref. 8) was similarly induced upon differentiation (Fig. 5*B*). Induction of *Cip1/WAF1* promoter activity was also observed after transient transfection of primary keratinocytes from homozygous p53-null mice (Fig. 5*B*). Taken together, these results indicate that *Cip1/WAF1* induc-

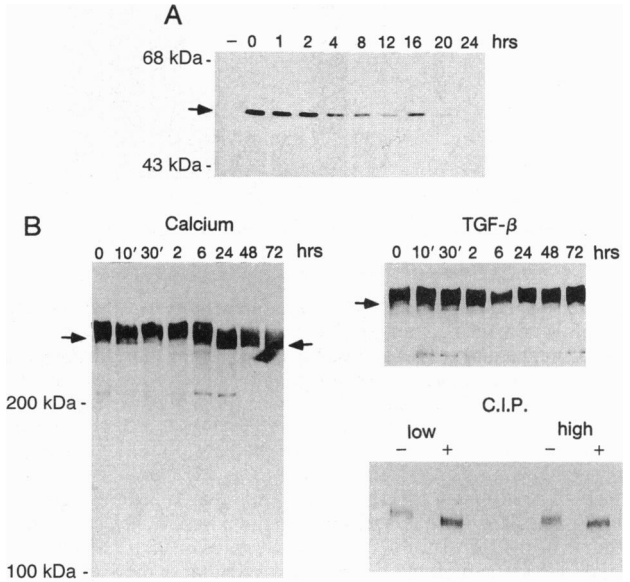

FIG. 4. State of p53 and p300 proteins in differentiating keratinocytes. (*A*) Immunoprecipitation with monoclonal antibodies against wild-type murine p53 of lysates prepared from ^{35}S-labeled primary keratinocytes under growing conditions (time 0) or at various times (in hours) after exposure to calcium (2 mM). Immunoprecipitation with an unrelated monoclonal antibody was used as a negative control (–). Immunoprecipitated proteins were separated on a SDS/10% polyacrylamide gel and visualized by autoradiography. The position of p53 is indicated by an arrow. (*B*) Immunoprecipitation with anti-p300 monoclonal antibodies of lysates prepared from ^{35}S-labeled primary keratinocytes under growing conditions (time 0) or at various times after exposure to calcium (2 mM) or TGF-β (10 ng/ml). Similar immunoprecipitates from keratinocyte lysates prepared from cells either in low-calcium medium (low) or switched to 2 mM calcium concentration (high) for 24 h were incubated with (+) or without (–) calf intestine alkaline phosphatase (C.I.P.). Immunoprecipitated proteins were separated on an SDS/5% polyacrylamide gel/0.17% bisacrylamide and visualized by autoradiography. Preliminary experiments showed that the broad band in the 300-kDa range (marked with arrows) was specifically immunoprecipitated by the p300-specific antibodies. A similar comigrating band was also found after immunoprecipitation of *E1A*-transformed Pam212 keratinocyte lysates (24) with anti-E1A antibodies. The weak band in the ≈200-kDa range is nonspecific, as it was detectable even after immunoprecipitation with antibodies unrelated to p300.

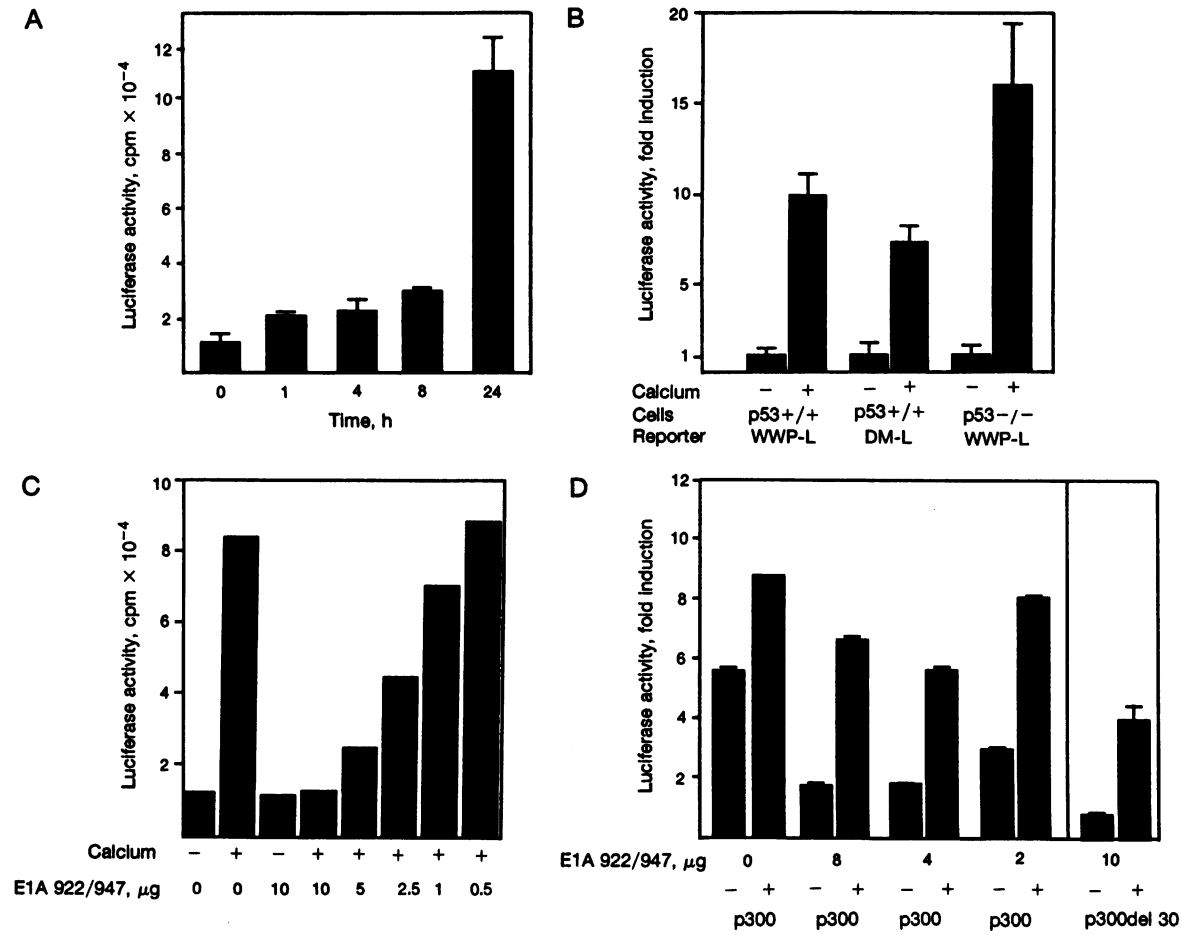

FIG. 5. Control of *Cip1/WAF1* promoter activity in differentiating keratinocytes. (*A*) Mouse primary keratinocytes in low-calcium medium were transiently transfected with a plasmid carrying the human *Cip1/WAF1* promoter (WWP-luc; ref. 8). Transfected keratinocytes were either kept in low-calcium medium (0) or exposed to high calcium concentrations (2 mM) for the indicated times (in hours) prior to termination of the experiment (72 h after transfection). All conditions were tested in duplicate samples and the experimental variation is indicated (bars). (*B*) Primary keratinocytes from wild-type (p53+/+) or p53-null mice (p53−/−) were transfected with the 2.4-kb *Cip1/WAF1* promoter (WWP-L) or a shorter fragment that lacks the fully conserved p53 recognition site (DM-L; ref. 8), as indicated. Transfected cells were either kept in low calcium medium (−) or switched to high calcium concentrations (+) for the last 24 h of a 72-h incubation period. (*C*) Mouse primary keratinocytes in low-calcium medium were transfected with the WWP-luc plasmid (5 μg per 100-mm dish) alone or together with decreasing amounts of the E1A 922/947 plasmid (26) as indicated. Transfected keratinocytes were either kept in low-calcium medium (−) or exposed to high calcium (2 mM) (+) for 24 h. (*D*) Mouse primary keratinocytes in low-calcium medium were transfected with the WWP-luc plasmid (2 μg per 60-mm dish) alone or together with decreasing amounts of the E1A 922/947 plasmid (26) plus or minus a cytomegalovirus expression plasmid carrying either a complete p300 cDNA (4 μg per 60-mm dish) or a mutated p300 cDNA which lacks the E1A binding region (p300del-30; ref. 16). Transfected keratinocytes were either kept in low-calcium medium or exposed to high calcium concentrations for 24 h. Similar results were obtained in four other independent experiments.

tion in differentiating keratinocytes is paralleled by increased promoter activity and is independent of p53 control.

Induction of *Cip1/WAF1* Expression in Keratinocyte Differentiation Is Dependent on the Transcriptional Modulator p300. The product of the adenovirus *E1A* oncogene interferes with cell growth and differentiation by binding and inactivating a number of cellular growth regulatory proteins (14). We tested whether E1A could interfere with the induction of *Cip1/WAF1* expression, as assayed by transient transfection of primary keratinocytes with the WWP-luc gene. Cotransfection of a fully transforming *E1A* gene or an *E1A* mutant able to bind to p105-Rb, p107, p130, or p60-cyclin A but not to the transcriptional modulator p300 (14, 24), drastically decreased the activity of the *Cip1/WAF1* promoter in keratinocytes irrespective of their growing or differentiating conditions (our unpublished observation). In contrast, an E1A mutant (E1A 922/947) which does not interact with the pRb family but still binds to p300 (14, 24) did not significantly affect the basal activity of the *Cip1/WAF1* promoter but suppressed its induction by calcium in a dose-dependent fashion (Fig. 5*C*). We tested whether this effect was due to binding of E1A to p300 by transfecting primary keratinocytes with the *E1A* gene with or without a cDNA for p300 driven by a cytomegalovirus promoter (16). Transfection of this cDNA alone had little or no effect on *Cip1/WAF1* promoter activity in keratinocytes under either growing or differentiating conditions (Fig. 5*D* and data not shown). In contrast, the ability of E1A 922/947 to suppress *Cip1/WAF1* promoter induction was reversed by the p300 cDNA (Fig. 5*D*). Such an effect was also observed with a mutated p300 cDNA lacking the E1A binding region (p300del-30; ref. 16; Fig. 5*D*). Thus, overexpression of p300 is sufficient to restore induction of the *Cip1/WAF1* promoter in differentiation.

In parallel with these findings, immunoprecipitation experiments revealed that the electrophoretic mobility of the p300 protein was increased after 6 h of calcium-induced differentiation (Fig. 4*B*). No such effect was observed after TGF-β treatment. The different migration rate of p300 immunoprecipitated from growing versus differentiating keratinocytes was abrogated by phosphatase treatment, suggesting that differential phosphorylation of p300 is involved (Fig. 4*B*).

DISCUSSION

Three major conclusions can be derived from the present work. Terminal differentiation in both keratinocyte and myoblast systems is associated with induction of *Cip1/WAF1* expression. In the keratinocyte system, induction of *Cip1/WAF1* expression is unlinked from p53 control. Finally, induction of this gene is dependent on the E1A-associated p300 protein.

Induction of *Cip1/WAF1* mRNA expression in terminally differentiating cells is accompanied by increases in $p21^{Cip1/WAF1}$ protein levels in parallel with a decrease of total CDK2 and p21-associated CDK kinase activities. $p21^{Cip1/WAF1}$-containing complexes exist in both an active and an inactive form, depending on their stoichiometry (5). Active complexes appear to contain a single molecule of this inhibitor, whereas inactive ones have multiple subunits (5). These observations, together with the fact that upregulation of the $p21^{Cip1/WAF1}$ protein was observed in both keratinocyte and myoblast differentiation systems, point to this inhibitor as a possible central player in cell-cycle control during differentiation. Future work will have to establish which additional regulatory mechanisms, such as CDK and cyclin modulation, contribute to CDK control during differentiation and whether they may function in a more cell-type-specific manner. However, besides being involved in CDK control, $p21^{Cip1/WAF1}$ could also inhibit DNA polymerase directly (6).

The onset of myoblast differentiation is associated with an increase of p53 expression (27), providing a possible mechanism for the observed *Cip1/WAF1* induction. In keratinocytes, p53 gene expression is decreased in response to calcium. More significant, *Cip1/WAF1* gene expression is induced by calcium even in the absence of p53. Absolute levels of *Cip1/WAF1* expression are lower in the p53-null keratinocytes than in the wild-type control. It will be interesting to determine the modality by which p53-null keratinocytes undergo growth arrest during differentiation, as cell-cycle control of these cells may be significantly abnormal (28).

Very little is known about control of the *Cip1/WAF1* gene, besides its being modulated by the p53 protein. Evidence for an as yet uncharacterized p53-independent pathway for upregulation of this gene in human cells has been recently presented (29). Indirect evidence has implicated the E1A-associated p300 protein in cell-cycle control and differentiation (refs. 15 and 16 and references therein). The effect on the *Cip1/WAF1* promoter provides the first direct evidence that p300 is involved in transmission of a differentiation signal linked to growth arrest. Suppression of the calcium responsiveness of the *Cip1/WAF1* promoter by E1A was counteracted to a similar extent by a wild-type p300 and a mutated form lacking the E1A-binding region. This indicates that the positive effect of p300 is not due to a functional sequestration of the E1A protein or simple competition for E1A binding to other p300-related proteins (30). In the absence of E1A, exogenous p300 was unable to stimulate *Cip1/WAF1* promoter activity to any significant extent in keratinocytes under either growing or differentiating conditions. Taken together, these observations suggest that the p300 protein is by itself not limiting but is required for other differentiation-related factors to function. An important role of p300 in keratinocyte differentiation is also suggested by the fact that this factor is also required for induction of the involucrin promoter, a well-characterized keratinocyte differentiation marker (ref. 31 and our unpublished observations).

Induction of keratinocyte differentiation is associated with specific protein phosphorylation events (ref. 32 and references therein). E1A has been shown to interfere with phosphorylation of the p300 protein (33), and the p300 phosphorylation state appears to be altered during calcium-induced keratinocyte differentiation. Future studies will have to address the complex interplay between protein phosphorylation, transcriptional control, and cell-cycle arrest in terminally differentiating cells.

Note Added in Proof. While this manuscript was under review, two similar reports of *Cip1/WAF1* induction in myoblast differentiation have appeared (34, 35).

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